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CONJUGATES OF TROLOX AND α-TOCOPHERYL SUCCINATE WITH NITROXIDES: SYNTHESSIS, ANTIOXIDANT AND ANTITUMOR ACTIVITY

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ABSTRACT

Two new derivatives of known antioxidant α-tocopheryl succinate - 4-carboxy-2,2,6,6-tetramethyl-4-(3-(3-oxo-3-((2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl)oxy)propyl)ureido)piperidine-1-oxyl (**1**) and 2,2,6,6-tetramethyl-4-(3-(3-oxo-3-((2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl)oxy)propyl)ureido)piperidine-1-oxyl-4-carboxylate, ammonium salt (**2**), as well as two derivatives of Trolox - 6-hydroxy-2,5,7,8-tetramethyl-*N*-(1-oxyl-4-amino-2,2,6,6-tetramethylpiperidine-4-yl)-chromane-2-carboxamide (**3**) and 6-hydroxy-2,5,7,8-tetramethyl-*N*-(1-oxyl-3-amino-2,2,5,5-tetramethylpyrrolidine-3-yl)-chromane-2-carboxamide (**4**) were synthesized. According to data of the SOS-chromotest and the Ames test all four compounds similarly to control Trolox and α-tocopheryl succinate (TSu) were not genotoxic and did not cause any damage leading to the block of DNA replication. Two derivatives **3** and **4** demonstrated 1.7-3.0-fold better antioxidant activity than Trolox. The protection of *S. typhimurium* (TA102) cells from *tert*-butyl hydroperoxide-dependent mutagenic effect by these compounds at low concentrations (0.3-3 μM) increased in the following order: **1** < **2** < Trolox < **3** < **4** ≤ TSu. In contrast to Trolox, all four derivatives (**1**-**4**) are not only antioxidants but also effectively suppress the growth of tumor cells: human myeloma, human mammary adenocarcinoma, and human hepatocarcinoma. The IC_{50} values depend significantly on the compounds (**1-4)** and type of tumor cells; except several cases they are varied mainly in the range 52-242 μM. Compounds **1**-**4** similarly to TSu were capable also to inhibit the growth of normal mouse (LMTK) and hamster (Ag17-1) fibroblast cells and demonstrate very different ratios in inhibition of various tumor and normal cell lines. Overall, compound **3** and **4** may be more promising antioxidants compared to Trolox.

KEYWORDS: Trolox, α-Tocopheryl Succinate, New Antioxidants, Inhibition of Tumor Cells Growth, Nitroxides

Abbreviations Used: Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TLC, thin layer chromatography; $IC_{50,}$ the drug concentration that caused 50% cell growth inhibition; ONPG, *o*-nitrophenyl-beta-D-galactopyranosidase; pNPP, *p*-nitrophenylphosphate; ROS, reactive oxygen species; t-BuO_2H, *tert*-butyl hydroperoxide; TSu, α–tocopheryl succinate

INTRODUCTION

The partially reduced oxygen species including $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$, that are produced as intermediates and by-products of aerobic respiration in all higher organisms and appear in cells through exposure to ionizing radiation, act as potent oxidants attacking different cellular DNA, proteins and lipids[1-4]. Ionizing radiation may cause damage to living tissue by producing free radicals like reactive oxygen species (ROS). ROS can randomly react with lipids, proteins and nucleic acids of cell causing oxidative stress and damage in these macromolecules, leading to different pathogenesis of chronic and age related diseases[1-9]. Central nervous system is very sensitive to oxidative stress, which is considered as a key factor of neurodegenerative disorders[10]. Oxidative stress is considered to be an important in pathogenesis atherosclerotic cardiovascular diseases[11], acute pancreatitis[12], diabetes and its complications[13], majorly deterioration of the skin morphology[14], and many other pathologies[5-9]. Inherited overgeneration of free radicals in animals is accompanied by a number of morbid conditions resembling human degenerative diseases of aging such as cataracts, cardiomyopathy, emphysema, carcinogenesis and others, as well as short life-span and low fertility[3, 5, 15, 16].

The first line of defense from the damaging effects of different radicals and ROS is antioxidants, which convert the oxidants to less reactive species and re-establish the equilibrium between pro- and antioxidants[10, 17]. A wide range of enzymatic antioxidants including antioxidative enzymes such as superoxide dismutase, catalase, glutathione peroxidase[3, 18], and mammalian catalytic antibodies with anti-oxidant activity[19, 20], present either naturally or supplied through food, are known to shield the effect of ROS generated during stress response.

Several natural antioxidants are known including flavonoids, phenolics, carotenoids[14], vitamins (A and C), vitamin E or its derivative *Adrusen Zinco* (vitamin E complex with zinc, copper, selenium and ω-3 polyunsaturated fatty acids), lipoic acid, glutathione, *Mirtilene Forte* (*Vaccinum myrtillus* extract)[21]. Natural antioxidants have no any side effects. At the same time, they are not universal in the case of different diseases and to some extent are organ specific. For example, *Mirtilene Forte* protects first of all the organs of sight and prevents the formation of cataracts[22].

Trolox, α-tocopherol (vitamin E), and α-tocopheryl succinate are the phenolic compounds with high antioxidative potential and they are perspective predecessors for the synthesis of new hybrid biologically active compounds with polyfunctional activity.

Trolox amides with carnosine have demonstrated both high-efficiency protection of brain neuron cells from oxidative stress as compared with initial/starting carnosine and higher antioxidant properties than the initial/starting molecules[23]. Apart from antioxidant properties, *in vivo* studies of amide conjugates of trolox with lipoic acids showed their antiarrhythmic features[24]. Biologically active trolox amides and amines have been synthesized, among which, for a large group of 2-aminomethylchromanes, a significant decrease of IC_{50} (up to 10 times) in comparison with α-tocopherol and trolox have been revealed[25]. N-ethylamide of trolox showing selective antiparasitic properties appeared to be a perspective treatment for leishmaniasis[26]. Besides, on the basis of trolox conjugates with triterpene acids of lupanic series, kojic acid and other phenolic acids, a series of new antioxidants have been synthesized[27]. Conjugate of trolox with dihydroxyphenyl propinoic acid – an inhibitor of tyrosinase and a strong antioxidant – has been detected, which is determined by the presence of resorcine and chromane fragments in the molecule[28].

α-Tocopheryl succinate belongs to the mitocan group i.e., mitochondrial targeted anticancer drugs[29]. This compound has high cytotoxicity and selectivity against various types of tumor cells (e.g. breast cancer cells, prostate

cancer cells, stomach cancer cells, melanomas, carcinomas, etc.)[30]. It has been demonstrated that TSu induces apoptosis of cancer cells in concentrations which are not toxic for intact cells and tissues[31]; it inhibits proliferative activity of cancer cells; strengthen other anticancer drugs[32], decreasing their toxicity against intact cells; does not have side effects, and in combination with vitamins C and К its anticancer activity increases[33]. On the basis of TSu, complex hybrid systems of target anticancer drug delivery have been created[34].

Synthesis and research of pharmacological properties of polyfunctional “hybrid” compounds containing fragments of nitroxyl radicals in the molecule (spin-labeled conjugates) is a rapidly developing area of medicinal chemistry [35, 36]. Spin-labeled analogs of biologically active natural compounds of different classes containing nitroxyl radicals are known: anthracycline antibiotics, lignans, triterpene acids, chromanes, flavonoids, stilbenoids, alkaloids, amino acids, etc.[36]. The introduction of nitroxyl fragment into a molecule leads sometimes to either strengthening of biological activity or its modification, decrease of general toxicity, or increase of selective cytotoxicity[35, 36].

The rationale to study new antioxidants is looking for derivatives of known substances with such properties having no toxic, mutagenic or carcinogenic properties. Such antioxidants are 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and □-tocopheryl succinate (TSu)[35, 37].

In this work, four new derivatives of Trolox and TSu (**1-4**) were synthesized according **Figure 1** and **2.** A possible ability of these compounds to protect bacterial cells from spontaneous and peroxide-induced mutagenesis and their cytotoxicity against cancer and normal cells were investigated and compared with that for Trolox and TSu.

MATERIALS AND METHODS

Materials, Chemicals

Reagents used in this work were obtained from Merck and Sigma Chemical Co. Tert-butyl hydroperoxide, and *p*-nitrophenylphosphate (pNPP), *o*-Nitrophenyl-beta-D-galactopyranosidase (ONPG) substrates, and Trolox were from Sigma (USA), ampicillin from AppliChem (Germany), diphenylphosphoryl azide from Acros Organics (USA). Bacto agar was from Difco (USA). TSu was prepared according to literature procedure[37].

The IR spectra were acquired on a Bruker Vector-22 spectrometer (Germany) in KBr and are reported in wave numbers (cm^{-1}). HRMS were recorded on double-focusing, high resolution mass spectrometer equipped with high performance toroidal ESA. The NMR spectra of solutions of the compounds in CD_3OD (δ, ppm; J,Hz) were recorded on a Bruker AV-400 spectrometer (Germany) with the working frequency of 400.13 MHz for ^1H NMR. ^1H chemical shifts (δ) were internally referenced to the residual solvent peak. Reactions were monitored by thin layer chromatography (TLC) on silica gel plates, visualizing with ultraviolet light. The products of the reaction mixtures were separated by column chromatography on Silicagel (230–400 mesh).

Synthesis of Nitroxides 1-4

TSu was prepared according to standard literature procedure by acylation of DL-□-tocopherol with succinic anhydride[45]. Unnatural amino acid spin label 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TOAC) was explored here for conjugation with TSu. On the first step TSu was converted to corresponding isocyanate using diphenylphosphoryl azide (Figure 1).

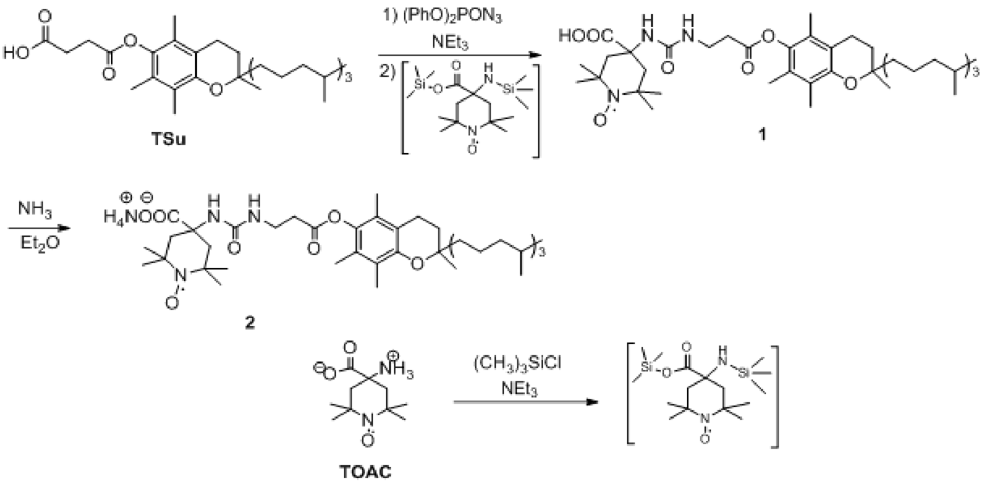

Figure 1: Scheme of the Synthesis of Compounds 1 and 2

Resulted isocyanate was added to trimethylsilyl ester of TOAC. The latter was obtained *in situ* by reaction with trimethylsilyl chloride.

Carboxyl function of TOAC allows saving good solubility of the resulting conjugate **1**. By treating of compound **1** with solution of gaseous ammonia in the ether ammonium salt **2** was obtained (Figure 1).

Trolox nitroxides **3** and **4** were prepared by reacting of Trolox with appropriate nitroxide: 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO) or 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-amino-PROXYL) in THF in the presence of *N*,*N*-carbonyldiimidazole (CDI) (Figure 2) according to a procedure described in[46]. The structures of the compounds **3** and **4** were confirmed by X-ray analysis, ^{13}C and ^{1}H NMR, EPR, IR, HPLC-MS methods[47].

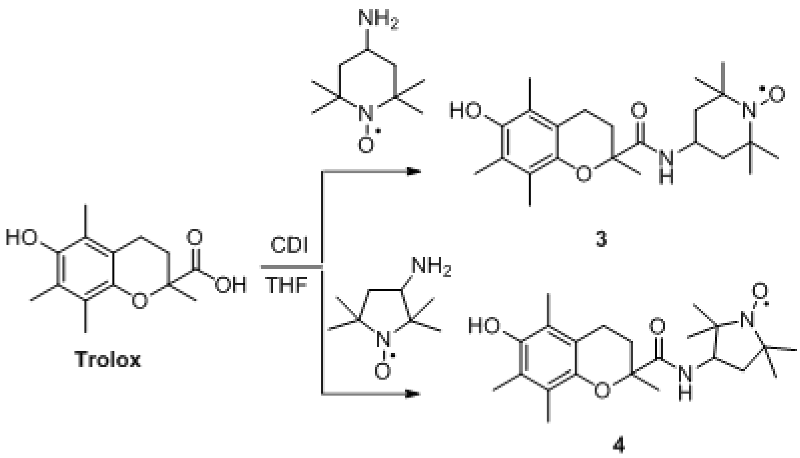

Figure 2: Scheme of the Synthesis of Compounds 3 and 4

Procedure for the synthesis of 4-carboxy-2,2,6,6-tetramethyl-4-(3-(3-oxo-3-((2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl)oxy)propyl)ureido)piperidine-1-oxyl (1). On the first step single neck 25-ml round-bottom flask equipped with $CaCl_2$ tube, condenser and magnetic stirrer bar was charged with DL-□-tocopheryl succinate (1 equiv, 0.4 g, 0.8 mmol), triethylamine (1 equiv, 150 µl, 0.8 mmol) and diphenylphosphoryl azide (DPPA) (1 equiv, 200 µL, 0.8 mmol). The resulted mixture was refluxed in dry benzene for 2 h. After that the solvent was removed under

reduced pressure and the residue (isocyanate of TSu) was used on the third step (see below). On the second step 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TOAC) (1 equiv, 0.2 g, 0.9 mmol) with trimethylsilyl chloride (2.5 equiv, 0.3 ml, 2.3 mmol) in 10 ml of dry benzene was heated at 40 °C for 2h. On the third step the suspension obtained was cooled to 0 °C and triethylamine (2.5 equiv, 335 µL, 2.3 mmol) and isocyanate of TSu were added. The reaction mixture was allowed to stay at ambient temperature for 24h. After that the reaction was completed (control by TLC, SiO_2, EtOAc + 0.5% CH_3COOH), the solvent was removed under reduced pressure, the residue was dissolved in H_2O (50 ml) and extracted with $CHCl_3$ (5×20 ml), the extract was dried over Na_2SO_4, the solvent was removed under reduced pressure, and the residue was separated by column chromatography (SiO_2, EtOAc, then EtOAc + 0.5% CH_3COOH) to give **1** as pale yellow glassy solid. Yield: 669 mg, 82%. mp 93–94 °C dec. IR (KBr) ν_{max}: 3367; 2951; 2927; 2868; 1753; 1743; 1718; 1645; 1562; 1462; 1377; 1244; 1163; 1109; 1080; 914; 667; 657. Anal. calcd for $C_{43}H_{72}N_3O_7$: C, 69.51; H, 9.77; N, 5.66; found: C, 69.35; H, 9.64; N, 5.48. HRMS EI (m/z) calcd for $C_{43}H_{72}N_3O_7$ 742.5322, found 742.5320. To confirm structure of nitroxide, the mixture of nitroxide **1** (0.33 mmol), activated zinc powder (1 g), and ammonium chloride (0.1 g) in methanol (10 ml) was stirred for 2 h at room temperature. The reaction mixture became colorless in the course of the reaction. The zinc excess was filtered off and the solvent was evaporated at a reduced pressure. The residue (the hydroxylamine) was used for NMR spectra recording. ^{1}H NMR (400 MHz; CD_3OD): δ 0.88/0.89/0.91 (3 × s, 3 × 3H, CH_3 from isoprenoid side chain), 1.15-1.59 (m, 24H, CH_3, CH_2, CH from isoprenoid side chain) 1.79−1.88 (m, 2H, CH_2), 1.97 (s, 3H, CH_3), 1.98 (s, 12H, CH_3 of hydroxypiperidine), 2.01 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.13 (d, 2H, J^2 = 15 Hz, CH_2 of hydroxypiperidine), 2.44 (d, 2H, J^2 = 15 Hz, CH_2 of hydroxypiperidine), 2.64 (t, 2H, J = 6.4 Hz, CH_2), 2.87 (t, 2H, J = 6.2 Hz, CH_2), 3.52 (t, 2H, J = 6.2 Hz, CH_2).

Procedure for the Synthesis of 2, 2, 6, 6-Tetramethyl-4-(3-(3-oxo-3-((2,5,7,8-Tetramethyl-2-(4,8,12-Trimethyltridecyl)Chroman-6-yl)Oxy)Propyl)Ureido)Piperidine-1-oxyl-4-Carboxylate, Ammonium Salt (2)

A solution of ammonia in dry ether was added dropwise to radical **1** in dry ester. After that the reaction was completed (control by TLC, SiO_2, EtOAc + 0.5% CH_3COOH), the reaction mixture became a gel. The solvent was removed under reduced pressure from the gel, the residue was glassy pale orange solid. Yield: quantitative, mp 78-79 °C dec. IR (KBr) ν_{max}: 3384; 3195, 2952; 2927; 2868; 1753; 1651; 1568; 1462; 1411; 1379; 1334; 1282; 1257; 1244; 1159; 1109; 1082; 1020; 937; 925; 804; 655; 617; 559. Anal. calcd for $C_{43}H_{75}N_4O_7 \times H_2O$: C, 66.38; H, 9.97; N, 7.20; found: C, 66.24; H, 9.79; N, 7.12.

General Method for Preparing Spin-Labeled Trolox Derivatives 3 and 4

A solution of trolox (80 mg, 0.32 mmol) in THF (0.65 ml) at 20–25°C was stirred, treated with CDI (65 mg, 0.34 mmol), stirred for 1 h, and treated with a solution of the appropriate radical (0.32 mmol) in THF (0.32 ml). The course of the reaction was monitored by TLC (Sorbfil, $CHCl_3$:EtOH, 9:1). The reaction time was 16–20 h. The solvent was evaporated. The solid residue was dissolved in CH_2Cl_2; rinsed successively with H_2O (3 ml), HCl (0.1 N, 2 ml), and saturated Na_2CO_3 solution (1.5 ml); dried over Na_2SO_4; and evaporated. Compound **3** was isolated by column chromatography over silica gel (EtOAc:hexane, 1:10) and subsequent crystallization from the same solvent. Compound **4** was isolated by column chromatography over reversed-phase sorbent Diapak C-16 (60% aqueous MeOH).

6-Hydroxy-2, 5, 7, 8-tetramethyl-*N*-(1-oxyl-4-amino-2, 2, 6, 6-tetramethylpiperidine-4-yl)-chromane-2-carboxamide (3). Orange crystalline compound. Yield 88 mg (68%), mp 65–66°C. UV spectrum (MeOH:H_2O, max, nm): 208, 292. Mass spectrum: found *m*/*z* 402.250 $[M – H]^{–}$, $C_{23}H_{35}N_2O_4$, calcd *m*/*z* 402.252 $[M – H]^{–}$. IR (KBr) ν_{max}: 3418, 2976, 2934, 1663, 1520, 1460, 1373, 1317, 1259, 1244, 1180, 1090, 1059, 991, 935, 862, 559.

6-Hydroxy-2,5,7,8-tetramethyl-*N*-(1-oxyl-3-amino-2,2,5,5-tetramethylpyrrolidine-3-yl)-chromane-2-carboxamide (4). Liquid viscous orange compound. Yield 70 mg (56%). UV spectrum (MeOH:H_2O, max, nm): 208, 292. Mass spectrum: found *m*/*z* 388.238 $[M – H]^{–}$, $C_{22}H_{33}N_2O_4$, calcd *m*/*z* 388.236 $[M – H]^{–}$. IR (KBr) ν_{max}: 3425, 2974, 2932, 1666, 1520, 1462, 1373, 1302, 1259, 1242, 1204, 1142, 1090, 1059, 937, 864, 575.

^{1}H and ^{13}C NMR data of reduced forms **3** and **4** are given in [47].

RESULTS

SOS-Chromotest

SOS-chromotest was carried out according to standard procedures[38], with some modifications [39, 40]. For the SOS-chromotest *E. coli* PQ37 strain (*sfiA*: Mud (Ap *lac*) *cts*, *lac*ΔU169, *mal*$^{+}$, *uvrA*, *galE*, *galY*, PhoC, *rfa*) provided by Kilarde F. (France) was used. The overnight culture of *E. coli* was grown to an optical density of 0.45 (2×108 cells/ml) on a shaker at 37°C. Then overnight culture was diluted 10-fold by LB. Aliquots of the suspension obtained 600 μl were added to tubes containing 10 μl of compound analyzed. Samples were incubated on a shaker for 2 h at 37°C and then the relative activities of β-galactosidase and alkaline phosphatase were analyzed.

For the analysis of β-galactosidase activity to 30 μl of every sample 270 μl of buffer A (0.2 M sodium phosphate, pH 7.75, 0.1 M KCl, 10 mM MgSO4, 0.3 mM DTT, and 0.1% SDS) were added and the mixture incubated at 37° C for 10 min. The reaction was started by addition of 60 μl of substrate ONPG (4 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0), and then incubated in at 37° C for 25 min. The reaction was stopped by adding of 200 μl of 1 M Na_2CO_3. The optical density (A_{405}) of solutions was measured.

For the analysis of alkaline phosphatase activity to 30 μl of every sample 270 μl of buffer B (1 M Tris-HCl, pH 8.05, 0.1% SDS) was added and this mixture was incubated at 37°C for 10 min. The reaction was started by adding of 60 μl of pNPP substrate (4 mg/ml in 1 M Tris-HCl, pH 8.05), and then the mixture was incubated at 37° C for 10 min. The reaction was stopped by addition of 200 μl of 1.5 M NaOH. The optical density (A_{405}) of solutions was measured. All compounds were dissolved in DMSO, which was used as negative control and t-BuO_2H as positive one. For evaluation of underground instead suspension in all experiments was used LB medium and DMSO. Estimation of relative activities (RA) of the enzymes was performed according to[39, 40]: RA = (1000 x A_{405}) / t (min) and average values were estimated. Then the ratio (R) of relative activities of β-galactosidase and alkaline phosphatase was calculated:

Ratio = activity of β-galactosidase (RA) / activity of alkaline phosphatase (RA). The factor of genotoxicity induction was calculated: R (compound) / R (control), where R (compound) is the ratio of the enzymes activities at one of compound concentration used, while R (control) is the ratio of the activities after cell treatment only with DMSO.

Determination of Mutagenicity of Compounds by Ames Test

In the Ames test, the histidine-dependent strain of *S. typhimurium* TA102 (rfa, +, + R) from collection of B. Ames (USA) was used, which carries a mutation at the histidine operon[41]. The mutagenic activity of the samples was analyzed

by the standard method without metabolic activation[41]. A liquid culture of TA102 was obtained by 16-h growth of cells from a frozen stock at 37°C in LB medium with penicillin. Then cells were plated on minimal glucose agar, antibiotics and histidine at the density sufficient to obtain isolated colonies. A separate bacterial colony was inoculated into LB medium (5 ml) containing ampicillin (50 μg/ml) and tetracycline (2 μg/ml), and grown with shaking (130 rpm) for 15 h at 37°C.

The Ames test was carried out using the described double-layer method[41, 42]. The overnight culture of bacteria (100 μl) after addition of analyzed compounds (in 10 μl DMSO) in different concentrations and t-BuO_2H in required concentration (if necessary) were mixed at 42°C with 2 ml of liquid 0.6% top agar. The mixture was poured onto plates with a minimal medium containing 0.2% glucose and 3% agar, taking care to distribute the mixture on the surface of the solid agar uniformly. The plates were incubated for 48 h at 37°C, and the revertants were counted. The cells incubated with t-BuO_2H in the absence of compounds analyzed were used as positive control, and the cells grown in the absence of t-BuO_2H and antioxidants (only with DMSO) served as negative control for the mutation induction. The results are expressed as mean ± standard deviation of at least 3 independent experiments.

In the study of antioxidant properties t-Student test was used according to[43, 44]. Measurement error was determined as the standard deviation from the mean with independent measurements of the same sample[44].

Cytotoxicity Assays

Tumor cell lines from human myeloma RPMI 8226, human mammary adenocarcinoma MCF-7, human hepatocarcinoma (HEP G2) and control normal cells: mouse fibroblasts LMTK and hamster (Ag17-1) fibroblast cells (~2000 cells per well) were incubated for 24 h at 37ºC in IMDM or RPMI 1640 medium (5% CO_2) and then were treated with different compounds. After 72 h of cell incubation, the relative amount of live cells was determined using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (a standard colorimetric MTT-test[44]) and the compound concentration that caused 50% cell growth inhibition (IC_{50}) was determined. The results are expressed as mean ± standard deviation of at least 3 independent experiments.

Biological Studies

Analysis of Potential Genotoxicity of New Compounds in SOS-Chromotest

It is known that some compounds with antioxidant activity at the same time may be polyfunctional and possess mutagenic or carcinogenic properties. Obviously, drugs are more successful when they are not mutagenic at least at the therapeutic concentrations. Therefore, we first estimated genotoxicity of four new compounds using SOS-chromotest and Ames test.

SOS-chromotest is known as a quantitative colorimetric method for analysis of genotoxic activity. This test is based on the induction of SOS response in *E. coli* cells leading to a number of features including damage or stop of DNA synthesis[39, 40]. It allows estimating the ability of different mutagens to induce the expression in *E. coli* the *din*-genes (genes inducible in response to DNA damage) by the determination of the β-galactosidase activity, which structural gene, lacZ, is under the control of a promoter of any *din*-gene[39, 40].

In the *E. coli* PQ37 strain used by us, the structural β-galactosidase gene (lacZ) is inserted under control of an inducible promoter *din*-gene *sfiA*; β-galactosidase activity is dependent on *sfiA* gene expression and induced in bacterial cells under the action of many genotoxic agents. After treatment of *E. coli* PQ37 cells by genotoxic compound the relative activity of β-galactosidase should be measured[38, 40].

In some doses genotoxic compounds can inhibit protein synthesis, which may lead to the underdetermination of β-galactosidase. Therefore, for results correction of the data a parallel determination of the activities of inducible β-galactosidase and constitutively expressed alkaline phosphatase is usually used. The ratio of β-galactosidase and alkaline phosphatase activities at the dose used to their relationship in the absence of the agent (factor of the induction of the gene lacZ expression) reflects the value of the damaging effect of compound analyzed[38-40].

Comparison of properties of known genotoxic *tert*-butyl hydroperoxide (t-BuO$_2$H), control Trolox and new compounds (solved in DMSO) at the final concentration of 30 mM was carried out (Table 1).

Table 1: Comparison of Genotoxicity of Trolox, α-Tocopheryl Succinate and their Derivatives Using SOS-Chromotest

Compound	Factor of an Induction of Gene lacZ Expression*
DMSO (solvent)	1.0
t-BuO$_2$H (3.5×10^{-4} M)	11.6±2.0**
Trolox	1.0±0.1
-Tocopheryl succinate	1.1±0.1
Compound **1**	1.0±0.1
Compound **2**	1.3±0.2
Compound **3**	1.4±0.2
Compound **4**	1.1±0.2

*Factor of an induction gene lacZ expression was calculated as the ratio of inducible β-galactosidase and constitutively expressed alkaline phosphatase activities after *E. coli* treatment with compounds analyzed to their relationship in the absence of the agents.

**Mean ± S.D. from three independent experiments; values are statistically different from the control with probability $P \geq 0.99$.

In contrast to t-BuO$_2$H (0.35 mM) demonstrating high value induction factor, Trolox and its derivatives even at their very high concentration (3 mM) did not induce the expression of the *din*-genes of *E. coli* (Table 1). In addition, none of the tested compounds did not change the alkaline phosphatase activity, which indicates the absence of their genotoxicity. It means that these compounds did not cause any damage leading to the block of DNA replication. Nevertheless, potential genotoxicity of compounds was estimated additionally using other known method, Ames test.

Analysis of Potential Genotoxicity of Compounds 1-4 Using the Ames Test

The *Salmonella typhimurium* TA102 strain containing a mutation in the histidine operon (hysG428) is often used both for evaluation of mutagenicity of different compounds and for detection of antioxidant properties, as judged from suppression of spontaneous mutagenesis in this strain and from a decrease in mutagenicity of oxidants, usually H_2O_2 or other peroxides[41]. The mutagenic activity of compounds **1-4** was estimated in the Ames test[41] using *S. typhimurium* TA102 as reported by Kemeleva *et al.*[42]. The mutation induction in the Ames assay is estimated by calculating the frequency of reversion from histidine auxotrophy to prototrophy in response to the substance under testing[41, 42].

α-Tocopheryl succinate, Trolox and derivatives **1-4** were compared with t-BuO$_2$H at the same final concentration, 30 mM, all compounds were solved in DMSO (Table 2).

The Ames test similarly to SOS-hromotest shows the absence of mutagenic properties in the case of compounds analyzed.

Table 2: Comparison of Genotoxicity of Trolox, -Tocopheryl Succinate and their Derivatives Using the Ames Test

Compound	Number of Revertants
DMSO	209±10*
t-BuO_2H (1 mM)	482±21
Trolox**	230±13
TSu	216±17
Compound **1**	230±19
Compound **2**	246±14
Compound **3**	218±10
Compound **4**	221±17

*Mean ± S.D. from four independent experiments; values are statistically different from the control with probability P ≥ 0.99.

** Trolox, TSu, and their derivatives were used in 30 mM concentration.

The Study of the Potential Antioxidant Activity of Compounds 1-4 Using the AMES Test

Some antioxidant compounds are known to efficiently decrease the mutagenic effect of H_2O_2, t-BuO_2H, and other various peroxide compounds(41, 42). In the Ames test, t-BuO_2H was added to TA102 cells at the 2 mM concentration and the analyzed compound concentrations varied (Figure 3). At low concentrations (0.3 μM) control compound Trolox suppressed the t-BuO_2H-dependent formation of mutants only for 8.5 % (decrease from 100 to 91.5 %) of revertants, but it was increased to 14.4 % at higher concentration (3.0 μM) (Figure 3). Interestingly, the effect of the protection from t-BuO_2H-dependent mutagenic effect for Trolox at 30 μM was slightly lower, 12.4 % (Figure 3).

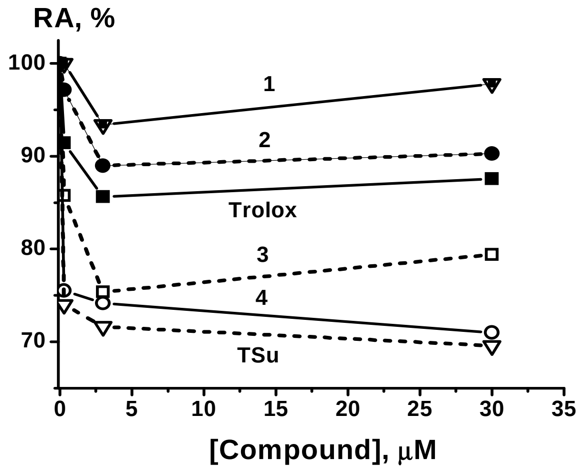

Figure 3: Analysis of the Antioxidant Activity of Four New Antioxidants on their Concentration by a Standard Ames test using the *S. Typhimurium* Strain TA102 in the Presence of 2 mM t-BuO_2H. The Number of Revertants in the Presence of t-BuO_2H was Taken for 100%. Used Antioxidants are shown on the Figure. The Average Error in Three Experiments for any Compound Concentration did not Exceed 5–10%

Similar situation was observed for **1**, **2**, and **3**, for which the decrease in protection effect at 30 μM concentration was observed, but this effect was remarkably higher for **4** and TSu at 30 μM than at 0.3-3 μM (Figure 3). Overall, the efficiency

of cell protection from effect of t-BuO_2H at low 0.3 and 3 μM concentrations of the compounds analyzed increased in the following order (%):**1** (0.0; 6.6) < **2** (2.8; 11.0) < Trolox (8.5; 14.4) < **3** (14.2; 24.6) < **4** (24.5; 28.4) ≤ TSu (26.0; 28.4) (Figure 1). It means that at low concentration (0.3 μM) compounds **3** and **4** are 1.7-3.0-fold better protectors for cells from t-BuO_2H-depndent mutagenesis than control antioxidant Trolox; **4** is the best antioxidant (Figure 3). Summarizing the data obtained, we can conclude that all new analyzed compounds are not genotoxic and/or mutagenic and possess antioxidant activities.

Assay of Cytotoxicity of Compounds 1-4 Forward Different Cells

It is known that some compounds can interact with many cell targets at the same time and possess not only antioxidant, but also cytoprotective and/or antitumor properties. Therefore, at the next step of evaluation of biological properties of new antioxidants, we have analyzed their ability to inhibit the growth of five mammalian cell lines; tumor cell lines from human myeloma (RPMI 8226), human mammary adenocarcinoma (MCF-7), and human hepatocarcinoma (HEP G2), as well as not cancer mouse (LMTK) and hamster (Ag17-1) fibroblast cells. Figure 4 shows representative data for compounds **3** and **4** in the case of MCF cells.

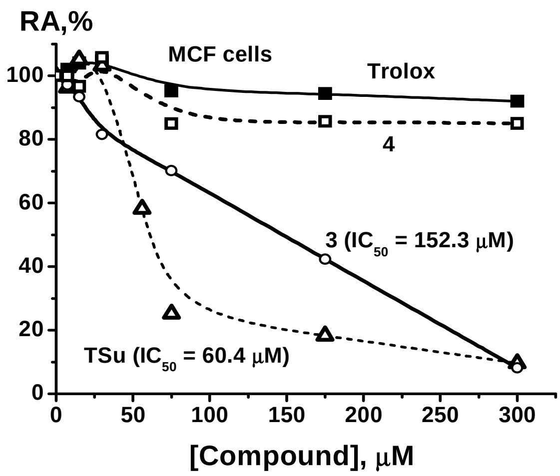

Figure 4: Effects of Several Compounds (Relative Activity, RA) on the Growth of MCF-7 Cells. The Average Error in Three Experiments for any Compound Concentration did not Exceed 5–10%

The results obtained for all new compounds with all types of cells are summarized in Table 3.

Table 3: Cytotoxicity (IC_{50}) of Different Compounds toward Various Tumor and Normal Cells

Compound	MCF-7	RPMI 8226	HEP	LMTK	AG-17
IC_{50} (μM) for Different Cell Lines					
Trolox**	n.d., >> 300**	n.d., >> 300	n.d., >> 300	n.d., >> 300	n.d., >> 300
TSu	60.4±6.0*	63.0±5.3	242.0±19.0	73.4±6.2	49.8±4.0
Compound **1**	58.0±4.8	81.5±7.0	n.d., >> 300	n.d., >> 300	185.4±16.1
Compound **2**	58.0±5.0	137.0±11.0	217.9±18.1	n.d., >> 300	63.2±4.9
Compound **3**	152.3±14.0	70.4±5.9	52.0±3.8	235.4±18.0	178.0±16.0
Compound **4**	n.d., >> 300	67.8±6.1	145.0±12.0	n.d., >> 300	190.0±16.4

*Mean ± S.E. from three independent experiments.

**When the I_{50} was higher than 300 μM, n.d. was used.

It can be seen that Trolox cannot effectively inhibit the growth all five types of cells even at very high concentration, 300 μM (Figure 2, Table 3). Similar situation was observed for compounds **1**, **2** and **4**; the concentration of these compounds caused 50% cell growth inhibition (IC_{50}) in the case of several types of cell lines were higher 300 μM: **4** (MCF, LMTK), **1** (HEP and LMTK), **2** (LMTK) (Table 3). All compounds, except **2** (IC_{50} = 137 μM) demonstrated the best inhibition of RPMI cancer cells (IC_{50} = 63.0–81.5 μM), but IC_{50} characterizing suppression of the growth of MCF and HEP tumor cells by these compounds varied in very wide ranges from 52 to higher 300 μM (Table 3). Figure 5 demonstrates the relative inhibition of the growth of all five types of cells used by all compounds at their 300 μM concentration. In contrast to antioxidant Trolox, all its derivatives are capable to suppress the growth of all five types of cells at high concentration (300 μM), but they demonstrate a very different effects of inhibition in the case of various cell lines. Tsu inhibits the growth of all types of tumor and normal cells remarkably more effective, than other derivatives.

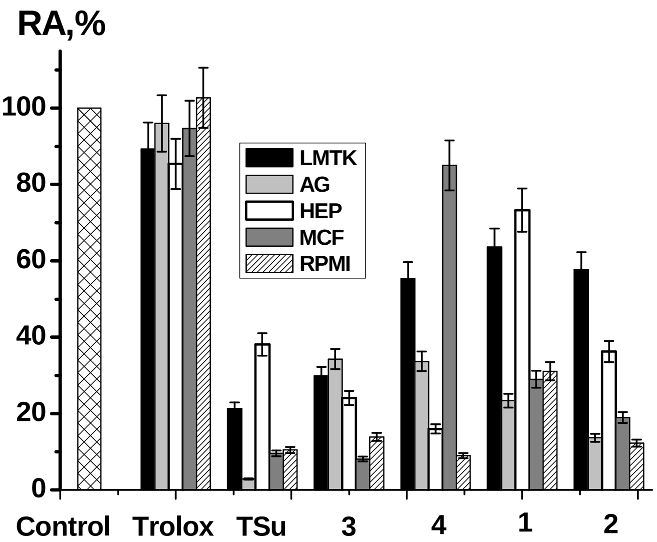

Figure 5: **Effects of Several Compounds (Relative Activity, Ra) at Concentration 300 μM on the Growth of Control LMTK and ag-17, as well as Tumor MCF-7, RPMI 8226, and HEP Cells. The Average Error in Three Experiments for any Compound did not Exceed 5–8%**

Antitumor drugs may be considered potentially more useful when they are better suppressors of tumor than normal mammalian cells. Therefore, it was interesting to compare the effects of these compounds on cancer cell lines and normal mouse (LMTK) and hamster (AG) fibroblasts. The best control antioxidant Tsu shows to some extent comparable IC_{50} values in the case tumor MCF, RPMI, normal LMTK, and AG cells (IC_{50} = 49.8-73.4 μM), while inhibition of tumor HEP cells is significantly worse (IC_{50} = 242 μM). Compound **3** better inhibits tumor RPMI and HEP cells (IC_{50} = 52.0-70.4 μM), but its efficiency in tumor MCF inhibition (IC_{50} = 152.3 μM) is comparable with that for AG (IC_{50} = 178 μM), but better than for LMTK (IC_{50} = 235.4 μM). Specific effective suppression of tumor RPMI cells was observed for compound **4** (IC_{50} = 67.8 μM), but this compound is bad inhibitor of all other cancer and non-cancer cells (Table 3). Compounds **1** and **2** are worse antioxidants than Trolox, but they are able effectively inhibit the growth of MCF cells. In addition, compound **1** suppresses the growth of non-cancer LMTK and AG cells at significantly higher concentrations (Table 3). Thus, several of these derivatives may be considered as specific inhibitors of some tumor cells in comparison with the normal ones, while TSu is completely unspecific suppressor of the growth of all type of cells.

DISCUSSIONS

Our data demonstrate that all new derivatives of Trolox and TSu are antioxidants showing no mutagenic and carcinogenic effects. Overall, the efficiency of cell protection from effect of t-BuO_2H at low concentrations of the compounds increased in the following order: **1** < **2** < Trolox < **3** < **4** ≤ TSu (Figure 3). Compounds **1** and **2** remarkably worse protect the bacterial cells from t-BuO_2H-induced mutagenesis than Trolox (Figure 3). Interestingly, control TSu demonstrates antioxidant activity, which is comparable with that for **4**. Compound **3** possess antioxidant activity intermediate between Trolox and TSu (Figure 3).

CONCLUSIONS

It is known that some antioxidants are useful for treatment of cancer patients. Interestingly, antioxidant Trolox cannot effectively inhibit the growth tumor and normal cells even at very high concentration, 300 μM (Table 3). In contrast to Trolox, new derivatives are not only antioxidants, but also are capable to suppress the growth of tumor and normal cells. All compounds demonstrate specific selectivity toward three type of tumor and two types of normal cells (Table 3). It is possible that all these compounds can play a double role, acting both as antioxidants and as selective inhibitors of some tumor cells growth. At the same time, significant inhibition of all types of tumor cells by all new compounds and control Tsu is observed at 10-100-fold higher concentrations than their antioxidant effect (Figure 3 and Table 3). Thus, they may first of all be considered as antioxidants and two of them (compounds **3** and **4**) are better antioxidants than Trolox.

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